STUDY OF PHENOTYPIC POLYMORPHISM AND DETECTION OF GENOTYPIC POLYMORPHISM IN *M. SEXMACULATUS* USING RAPD PCR

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ABSTRACT

Menochilus sexmaculatus (Coleoptera: Coccinellidae), commonly known as six spotted zig zag ladybird is aphidophagus used as an economically significant biological control agent of pest insects, including aphids. M. sexmaculatus is the most misidentified Coccinellid due to the occurrence of its numerous color variants. The correct identification of M. sexmaculatus and its strains is necessary to implement the use of biological control. In this study, phenotypic and genotypic polymorphism was investigated in Menochilus sexmaculatus collected from Punjab, Khyber Pukhtoonkhwa (KPK) and Sindh provinces of Pakistan. Six different morphs of the species were distinguished by analyzing its elytral color and spot pattern, and then Polymerase Chain Reaction (PCR) was used to generate random amplification of polymorphic DNA (RAPD) from six different types of M. sexmaculatus. Forty primers (OPA & OPC Kit) were used to perform RAPD PCR on six different types of M. sexmaculatus, out of which seven primers revealed different patterns related to the M. sexmaculatus types. These seven primers produced 111 clear polymorphic bands and 6 scorable strain specific markers. The cluster analysis applied to RAPD data showed high polymorphism among six types and it can be concluded that these six types are six polymorphic strains of the same species, which has implications for natural and biological control of aphids.

Keywords: *Menochilus sexmaculatus, aphidophagus, coccinellids, phenotypic and genotypic polymorphism, RAPD-PCR, strain specific markers*

1. INTRODUCTION

M. sexmaculatus commonly known as six-spotted zig zag ladybird widely distributed in Pakistan (Hamid, 1983). *Cheilomenes sexmaculata* and *Menochilus quadriplagiatus* are synonymous scientific names of this species (Sasaji and Akamatsu, 1979). *M. sexmaculatus* is a common species used for biological control in agriculture settings, as its main prey is the aphid – a major agricultural pest. The ability of *M. sexmaculatus* to be so successful in a large range of habitats makes it especially beneficial to humans who need crop security from aphid infestations. *M. sexmaculatus* is highly polymorphic. The various color morphs of this species are frequently misidentified as *Micraspis discolor*, and *Chilocorus nigrita*.

Morphology-based identification and description of insect taxa has long been used to separate species. Among many groups of insects, however, morphological characters within a single species can vary with respect to environmental factors, or on the other hand, related species may have convergent and cryptic morphologies which limits its usefulness. Under such conditions, studies of their biology and molecular profiles become essential for defining species and characterizing populations (Calvert, et al., 2001). Species that exhibit visible polymorphisms are ideal for studying the micro-evolutionary forces that maintain genetic variation in nature (Ekendall and Johansson, 1997). Many coccinellid species exhibit striking elytra color polymorphisms and so provide an excellent system to study questions about the evolution and maintenance of polymorphisms (Kearns, et al., 1990; Bernstein and Bernstein, 1998; Machado and Arjauno, 1999; Machado, et al., 2004).

Molecular methods have revolutionized insect systematic (Roderick, 1996; Caterino, et al., 2000) and are increasingly being applied to coccinellids (Navajas and Fenton, 2000). The ability to amplify DNA via the PCR has greatly facilitated DNA sequence comparisons (Innis, et al., 1990) and resulted in development and use of species diagnostic PCR primer pairs (Paskewitz, and Collins, 1990). The technique, random amplified polymorphic DNA (RAPD), is PCR based, permitting scores of markers to be assayed on DNA extracted from single insect. Instead of using primer pairs as in traditional PCR, RAPD reaction use a single short primer (usually ten bases in length) of randomly chosen sequence. RAPD PCR analysis increases the resolution of genetic differences. This method has been widely used for analysis of genetic variation of insect population and for identification of cryptic insect species and biotypes (Gawel and Bartlett, 1993; Black and Du Teau, 1997). The purpose of this study is to determine intraspecific phenotypic and genotypic differences of *M. sexmaculatus* by morphological and RAPD analysis, and identify a set of primers specific to the *M. sexmaculatus* genome.

2. MATERIALS AND METHODS

2.1 Morphological Study

Specimens of *M. sexmaculatus*, manually collected

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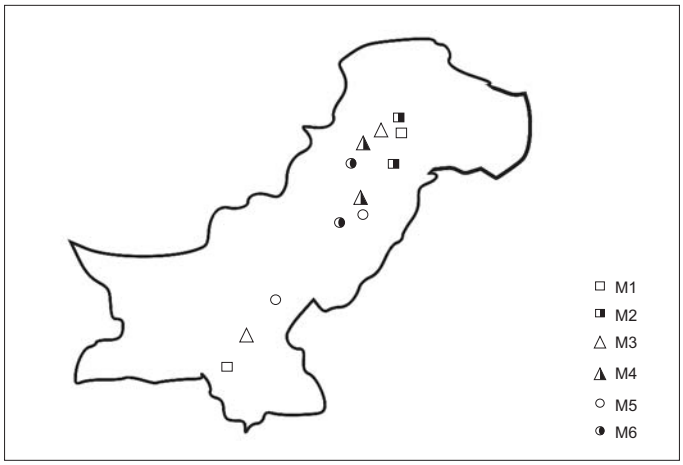

Figure-1: Map Showing the Location of the Sampling Sites of Different Types of *M. sexmaculatus*

Table-1: Details of *M. sexmaculatus* Specimens

Type of Specimen	Area	Province	Date of Collection
M1	Malir, Karachi	Sindh	Mar, 2007
	Phhagwari, Kotli	AJK	Oct, 2007
M2	Islamabad	Federal Area	Mar, 2002
	Rawalakot	AJK	Apr, 2006
M3	Umar Kot	Sindh	Mar, 2007
	Mangla	AJK	Apr, 2007
M4	Jhang	Punjab	Jan, 2008
	Mirpur	AJK	May, 2004
M5	Jhang	Punjab	Oct, 2006
	Sukhur	Sindh	Oct, 2003
M6	Tarbela	KPK	Sep, 2006
	Talagang	Punjab	Mar, 2004

from 11 different localities in three provinces of Pakistan, including KPK, Punjab and Sindh, were obtained from the National Insect Museum, National Agricultural Research Centre (NARC), Islamabad, Pakistan (Figure-1). The types of the specimen, and their date of collection are enlisted in Table-1. Different types of *M. sexmaculatus* were separated phenotypically by observing their elytral coloration and spot pattern, size, mouth parts and genitallium (Table-2).

2.2 Extraction of DNA

DNA was extracted from the individual insects by using two different protocols, namely DNA ZOL (MRC. Inc) and CTAB (Doyle and Doyle, 1990)

2.2.1 DNA ZOL

In this method, the whole single individual was lysed and homogenized with 1 ml of DNA ZOL (MRC Inc). Homogenate was stored for 5-10 minutes at room temperature. It was then sedimented for 10 minutes at 10,000 g at 4-25°C. Following centrifugation the resulting viscous supernatant was transferred to a fresh-labeled eppendorf tube. Afterwards, DNA was precipitated from the homogenate by adding 0.5 ml of 100% ethanol and stored at room temperature for 1-3 minutes. Precipitated DNA was again centrifuged at 10,000 g for 5 minutes at 4-25°C. Supernatant was removed simply by decanting. Precipitated DNA was then washed twice with 1 ml of 75% ethanol and suspended for 5 minutes. Remaining alcohol was removed by pipetting and the pellet was dissolved in 50 μl DEPC H_2O.

2.2.2 CTAB Method

CTAB method of Doyle and Doyle (1990) was followed with minor modifications. Individuals were separately

homogenized in 100 µl of Extraction Buffer (50 mM Tris pH 7.0, 100 mM NaCl, 10 mM EDTA, 1% SDS) followed by addition of 500 µl of TBE Buffer, mixing and incubation at 65°C for 1 hr in water bath. Afterwards, 400 µl of phenol (pH 8.0) along with 200 µl chloroform (1:24 Isoamyl alcohol: chloroform) was added to this solution, and centrifugation at 13,000 rpm for 8-10 minutes was carried out. The top clear solution was pipetted out into another clean-labeled eppendorf tube and 500 µl of chloroform was added. It was again centrifuged at 13,000 rpm for 8-10 minutes. Again top clear solution was pipetted out into another labeled eppendorf tube and mixed with 1/10 vol. of 3M Na_2 Acetate and 2 vol of 100% ethanol and centrifuged at 13,000 rpm for 8 minutes. Supernatant was discarded and 700 µl of 70% ethanol was added to wash pellet and then centrifuged at 13,000 rpm for 2-5 minutes. Supernatant liquid was discarded and dry pellet was re-suspended in 50 µl of DEPC H_2O and stored at –20°C. The concentration of DNA samples was quantified by UV spectrophotometry. Working solutions were adjusted to 25-30 µg/µl in DEPC H_2O.

2.3 RAPD PCR

A total of forty primers of random sequence from two kits (OPA and OPC, Operon technologies, Almaeda, USA) were used for amplification of DNA. PCR reactions were conducted with 25 µl reaction mixture. The optimum RAPD PCR reaction conditions were selected by varying several parameters viz. DNA concentration (10, 15, 20, and 25 ng); $MgCl_2$ concentration (1.5, 2, 2.5, 3, 3.5 and 4.5 mM); Primer concentration (0.1, 0.2, 0.3, 0.4, 0.5 and 0.6 µM) Taq DNA polymerase concentration (0.5, 1.0, 1.5 and 2.0 U) and dNTP concentration (2, 10, 20, 25, 50, and 100 mM).

The optimum reaction mixture that gave best amplification pattern was obtained using 2.5 µl of 10 X PCR buffer, 3 µl of $MgCl_2$ (2 mM), 1 µl of 10 mM dNTP's, 1 µl of primer (0.2 µM), 0.25 µl of Taq Polymerase (1 U), 1 µl of DNA (15 ng) and the rest DEPC H_2O.

Amplification was carried out in Reacon Thermal Cycler programmed for denaturation at 94°C for 1 min, 45 cycles of denaturation at 92°C for 1 min, annealing at 35°C for 1 min, extension at 72°C for 1 min and then final extension at 72°C for 10 min. Ten micro litres (10 µl) of amplification products were run along with a Gene Ruler™ DNA Ladder Marker (1 Kb) on 1% agarose gel containing Ethidium Bromide for 3 hrs at 40-50 V. Each set of PCR reactions was checked for contamination by using a negative control (all reagents except template DNA).

2.4 Cluster Analysis

RAPD bands produced by all the primers were scored for six strains. A matrix was created by taking into account the presence (1) or absence (0) of the bands. Using this matrix, polymorphism within the species of *M. sexmaculatus* was calculated (an index of Genetic distance 1-F values) using the formula of Nei and Li, (1979). Dendogram was constructed using UPGMA computational programme (POP Gene Software).

Table-2: Details of Six Morphologically Different Types of *M. sexmaculatus* used

Type of Species	Total Body Length (mm)	Pronotal Length (mm)	Pronotal Width (mm)	Elytral Length (mm)	Elytral Width (mm)	Ground Color	Elytral Pattern	Spot/Stripe Color
M 1	4.61	1.44	2.73	3.17	2.32	Dark Orange	Stripped	Black
M 2	4.64	1.47	2.8	3.17	2.38	Black	Stripped	Light Orange
M 3	4.32	1.2	2.6	3.12	2.24	Black & Yellow	Stripped	Black
M 4	4.02	1.14	2.32	2.88	2.16	Black	-	-
M 5	4.57	1.42	2.72	3.15	2.3	Dark Orange	Stripped	Black
M 6	4.52	1.39	2.7	3.13	2.3	Black	Spotted	Dark Orange

3. RESULTS

3.1 Phenotypic Polymorphism

Six different morphs of *M. sexmaculatus* showed slight variation in their elytral color intensities and size. Table-2 lists all the measurement and the basic color pattern of six types of *M. sexmaculatus*.

Six different morphs of *M. sexmaculatus* included in the study were obtained on the basis of elytral spot or stripe patterns. The elytral pattern of six different morphs of *M. sexmaculatus* are shown in Figure-2. Morphologically different specimens of *M. sexmaculatus* showed the same basic pattern in mouthparts. Wide massive and sickle-shaped mandible was observed having one apical and one basal tooth, which is characteristic of all predatory coccinellids (Figure-3 A). All the types carried a small rounded side labrum with setae on it (Figure-3B). Maxillary palpi were found four segmented. The terminal segment of *maxillary palpi* is an important distinguishing characteristic for identification of different types of *M. sexmaculatus* because of its unique axe-like shape (Figure-3 C). Labium is not taxonomically important and it was observed that numerous hairs anteriorly covered the labium and labial palus was three segmented (Figure-3 D).

Generally, species can only be identified with reference to their species-specific structure of male genitalia (Kovar, 1996), in the absence of other external diagnostic characteristics. Such diagnostic characteristics in the male genitallium are found in aedeagus (tegmen) and sipho. The specimens of *M. sexmaculatus* showed an elongate, curved and

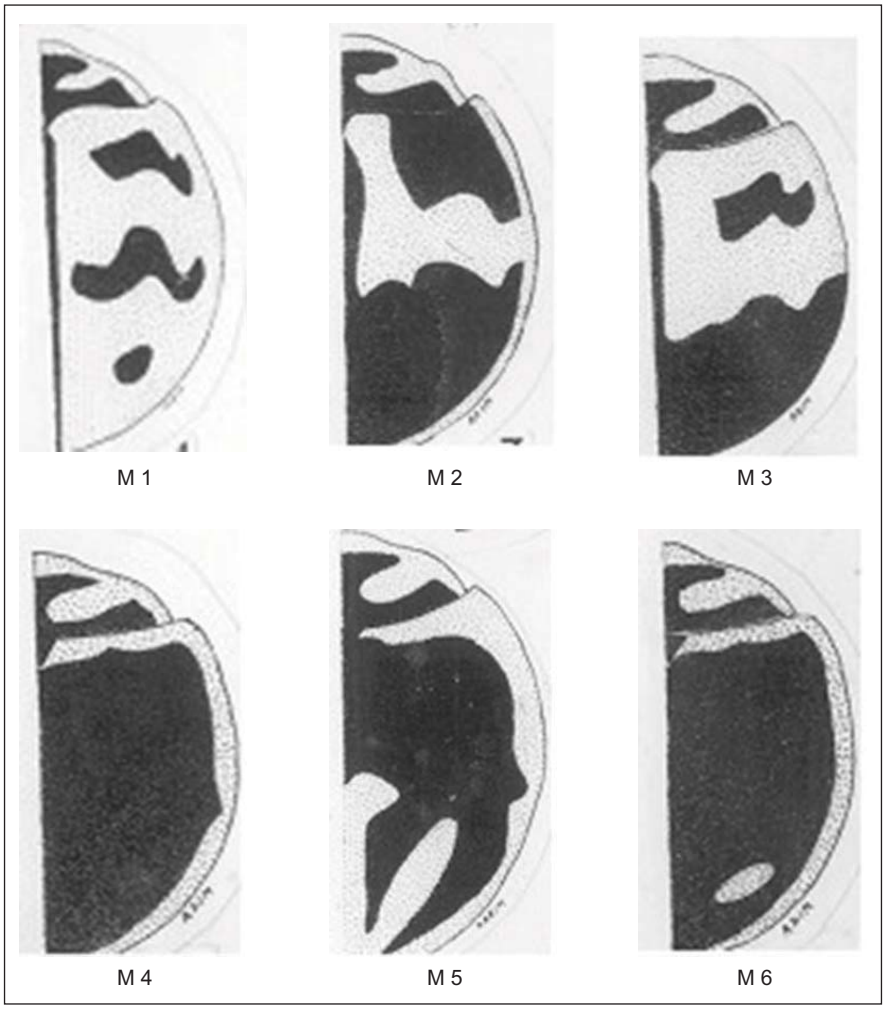

Figure-2. Elytral Pattern in *M. sexmaculatus*

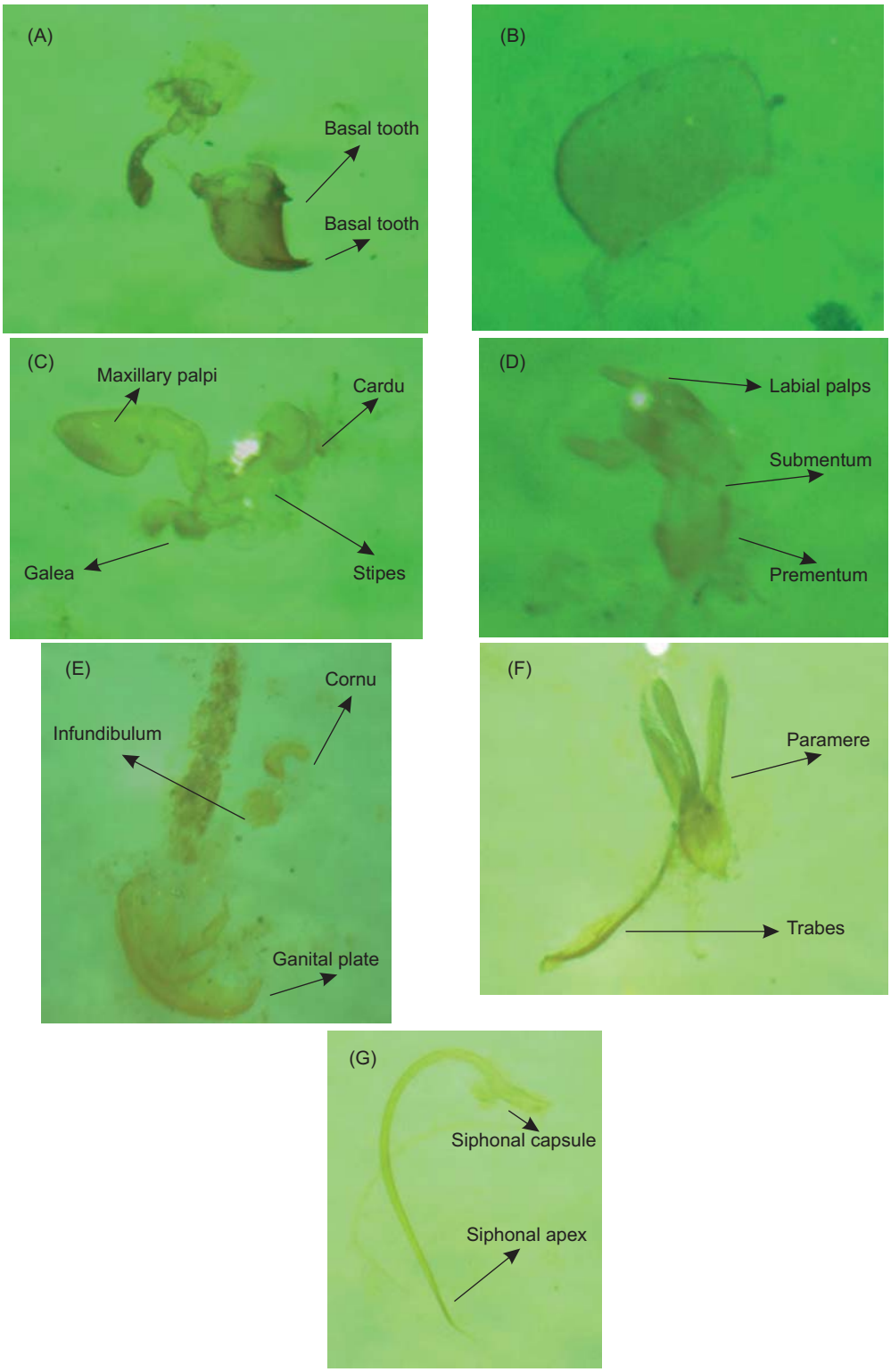

Figure-3: Phenotypic Characteristics of *M. sexmaculatus:* (A) Mandibles and Palp; (B) Labrum; (C) Maxilla; (D) Labium; (E) Female Genitallium; (F-G) Male Genitallium

tubular sipho with a basal siphonal capsule (Figure-3. F-G). All types had a symmetrical tegmen comprising of pair of parameres (arms). The shapes and structure of the genital plates of female genitallium are of taxonomic importance at the higher level. Phenotypically similar female genitallium was found from M1 to M6. All carried long and broad spermatheca (Figure-3 E).

3.2 Standardization of DNA Isolation

In this study, DNA ZOL method proved more efficient as compared to CTAB method. Several modifications were introduced to Doyle and Doyle (1990) CTAB method for the removal of the impurities. Use of CTAB method for DNA extraction resulted in sheared bands on gel electrophoresis indicating higher proteins. Isolation of DNA of *M. sexmaculatus* from the CTAB method resulted in insufficient removal of protein and viscous DNA samples. Two consecutive washes with Phenol chloroform (isoamyl alcohol) excluded protein impurities (Chakraborti, et al., 2006) and proved very effective. The pelleted DNA obtained by DNA ZOL method generated clear solution whereas the DNA isolated by the CTAB method was partially soluble. DNA ZOL proved to be more effective in removing the total protein content and for the recovery of pure DNA. Spectrophotometric values (260/280) for DNA extracted from six types of the species by DNA ZOL ranged from 1.45 to 1.66 while for C-TAB values ranged from 1.24 to 1.88.

3.3 RAPD PCR Amplification

The protocol of RAPD PCR was optimized by introducing several modification to the original Williams, et al. (1990) protocol in both PCR components, such as template DNA, Primers, magnesium chloride, Taq Polymerase, dNTP's, as well as in amplification cycles including, temperature and time interval for denaturation, annealing and extension steps. The optimized reaction conditions produced clear, scorable amplified products, suitable for RAPD applications in all primers tested.

Of the 40 primers of OPC-kit and OPA-kit used for amplification, 7 primers (OPA-04, OPA-09, OPA-18, OPC-04, OPC-12, OPC-15 and OPC-18) proved successful in detecting polymorphism in all types of *M. sexmaculatus* (Table-3). Out of the total 153 fragments scored, about 72% (111 bands) were polymorphic, while 42 fragments were monomorphic (shared by all strains). There were some fragments, which are species specific and are not shared by strains. The occurrence of common and unique bands between six types is valuable and can be used to gain phylogenetic information. The number and size of amplified products varied depending upon the sequence of random primer and DNA samples used. The size of amplified products ranged from 340 bp to 1200 bp.

The primer OPA 04 produced a total of 27 bands of different sizes with all types of *M. sexmaculatus* ranging from 350 bp to 940 bp in length (Figure- 4 A). Since one band was shared by all types (500 bp), therefore it is considered a marker for entire species. As one specific band of 880 bp was obtained for M 4 and did not appear in other types, it can be used as strain specific molecular marker for M4 strain. The primer OPA-09 amplified 17 bands with all types of *M. sexmaculatus* of sizes between 590 bp to 1030 bp in length. This primer cannot be used to distinguish the species, however the profiles show a large degree of variation, therefore it can be a good strain marker for conformation purposes (Figure-4 B).

The primer OPA 18 yielded a total of 22 bands with all types of *M. sexmaculatus*. The range of the bands lies between 340 bp to 1120 bp in length (Figure-4 C). RAPD PCR analysis for primer OPA 18 was to some extent similar to that of primer OPA 09. All the types of *M. sexmaculatus* when amplified with OPC-04

Table-3: Details of the Polymorphism Detected by the Primers

Primers	Primers Seequence	Total Bands	Polymorphic Bands	% of Polymorphic Bands
OPA-04	5'-CCGCATCTAC-3'	27	21	77.7
OPA-09	5'-GGGTAACGCC-3'	17	17	100
OPA-18	5'-AGGTGACCGT-3'	22	22	100
OPC-04	5'-CCGCATCTAC-3'	21	3	14.3
OPC-12	5'-TGTCATCCCC-3'	26	26	100
OPC-15	5'-GACGGATCAG-3'	23	5	21.7
OPC-18	5'-TGAGTGGGTG-3'	17	17	100

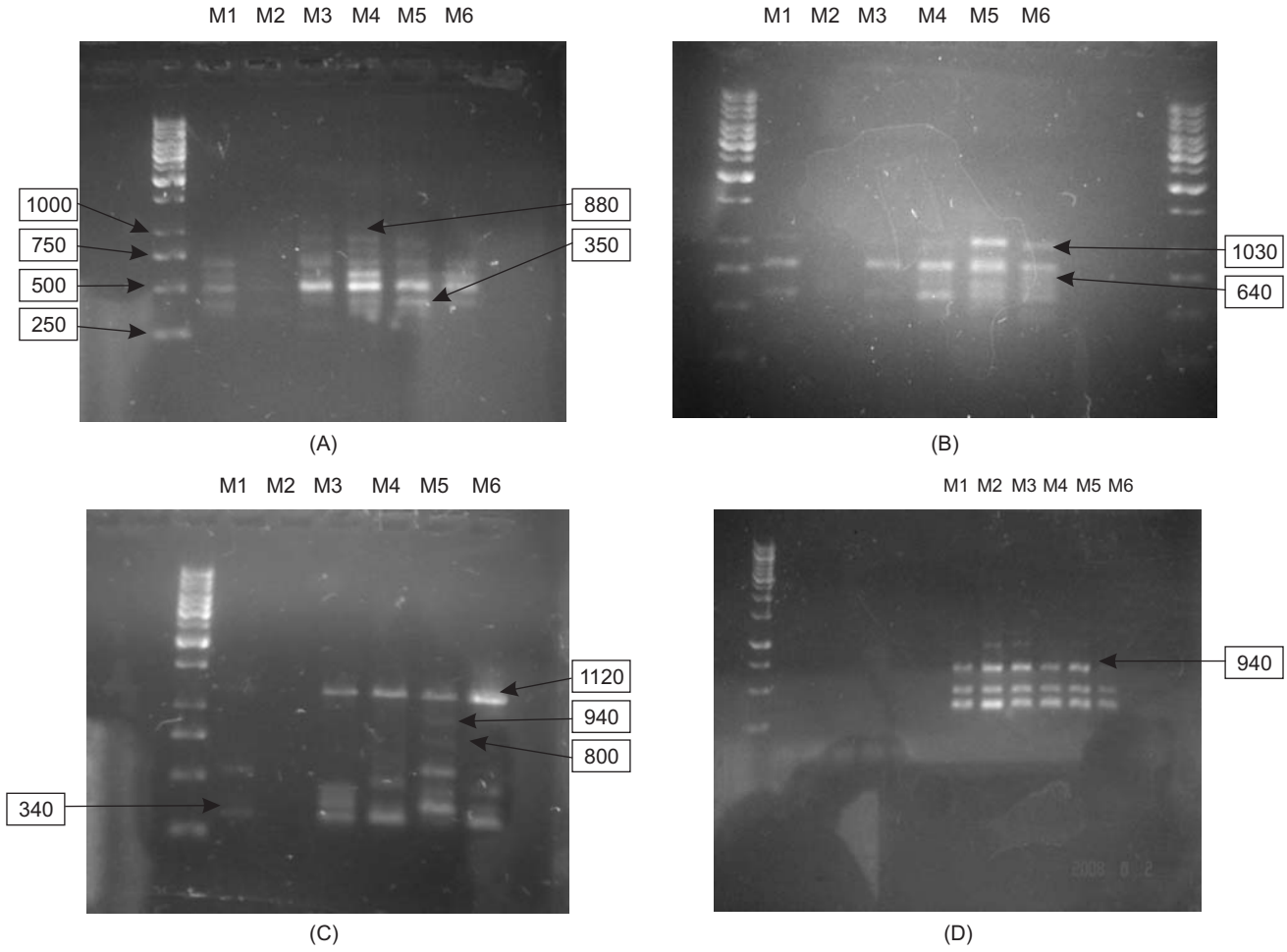

Figure-4: RAPD Reaction Products: (A) for OPA-04; (B) for OPA-09; (C) for OPA-18; and (D) for OPC-04

showed a total of 21 bands of sizes ranging between 360 bp to 940 bp in length (Figure-4 D). Successful detection of 3 common bands of 360 bp, 500 bp and 650 bp in all the types by OPC 04 primer indicates that this primer can be used as molecular marker for *M. sexmaculatus*.

A total of 26 bands of sizes ranging between 380 bp to 1060 bp in length were obtained when all types of *M. sexmaculatus* were amplified with OPC-12 (Figure-5 A). The DNA fragment profile of amplification with OPC 12 did not show a common band for all types but provided 2 markers that can be an excellent tool for fast and accurate identification of M 2 and M 5 types. M 2 differs from others types only in the position of one band of 380 bp, and M5 differs from others by the presence of one band (500 bp).

A total of 17 bands, ranging from 500 bp to 1200 bp in length were recovered by the primer OPC-18 with types of *M. sexmaculatus* (Figure-5 B). Single band of 1200 bp was observed only in M3 type, thus distinguishing it from all other strains and can be considered as useful strain-specific molecular marker. A specific band of 900 bp was recorded in M5 type so it can also be used as molecular marker for its identification.

Analysis of RAPD PCR for the OPC-15 primer (Figure-5 C) revealed a total of 23 bands. The range of the bands length was between 380 bp to 900 bp. Bands of 380 bp, 500 bp, and 680 bp were common to all the types. A band of 900 bp was commonly shared by all strains except M6. No specific band was observed in any strain. Three common bands that are shared by all strains can be used as species-specific molecular marker.

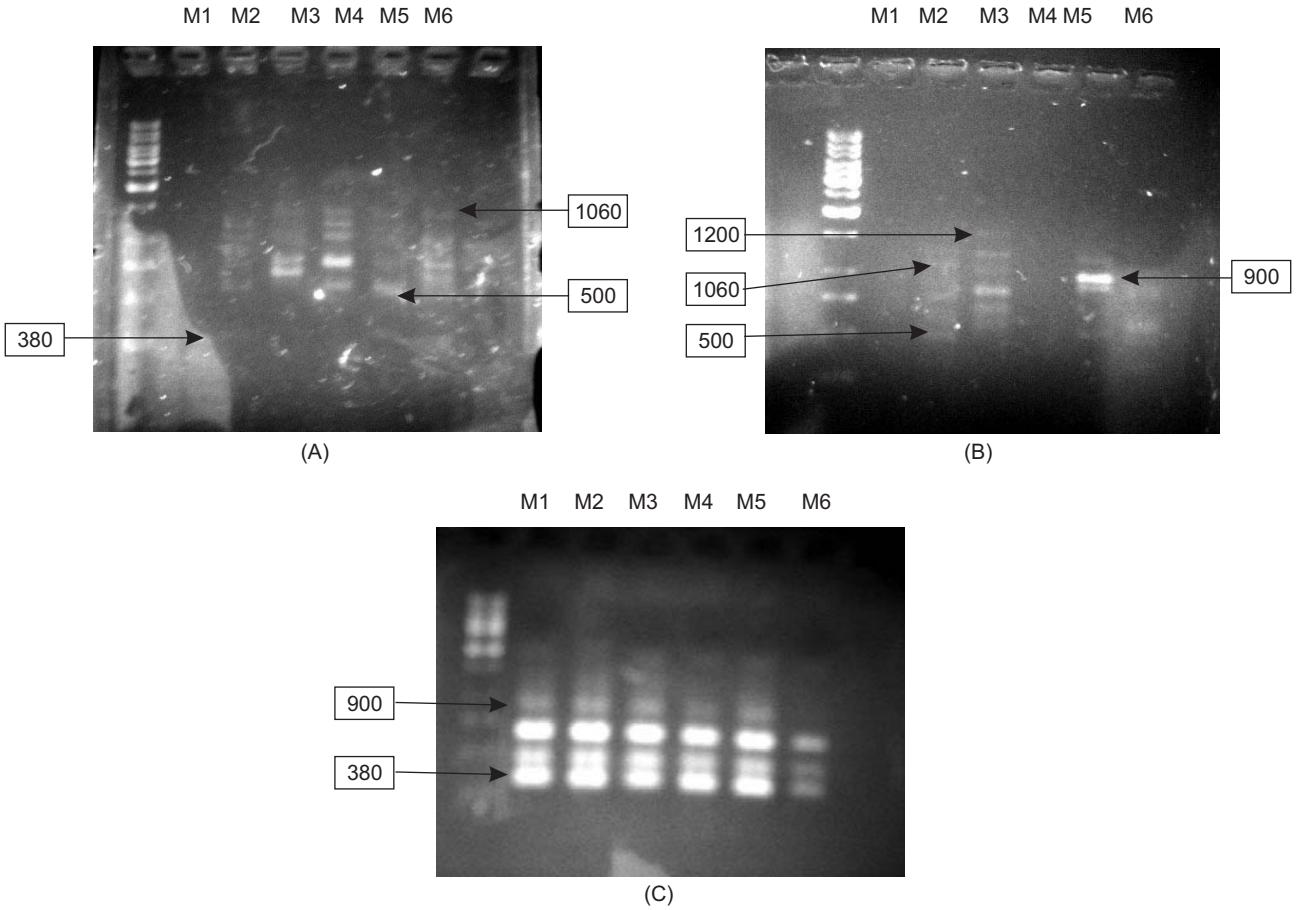

Figure-5: RAPD Reaction Products: (A) for OPC-12; (B) for OPC-18; and (C) for OPC-15

3.4 Dendogram

A dendogram was constructed based on the RAPD data generated in this study (Figure-6). The analysis was based on the number of bands that were different between any types. Dendogram divided six types of *M. sexmaculatus* into three main groups. First group contains single type M5, second contains M2 and third group contains the remaining types. Third group is further divided into two sub-groups. First sub-group encompasses M1 and second sub-group is further divided into two lineages or clades, one is composed of M3, and second one includes M4 and M6 types. Thus M4 and M6 are more closely related to each other than to any other types and M3 is closely related to these two types. At the same time, these three types showed slightest relationship with type M1. The least genetic distance of 0.2231 was recorded between M4 and M6, while the highest value of 0.6444 was between M5 and M6 (Table-4).

The results showed high association between genotypic and phenotypic polymorphism. The first group containing M4 and M6 strains differed only in the presence of one yellow spot on elytra, the rest of the elytral color pattern was the same. The cluster analysis done on the basis of RAPD data showed the least genetic distance between these two types. Then M1 and M3 resembles more to these two types phenotypically than M2 and M5. Type 1 and type 3 differed in color pattern below middle lateral part of the elytra and have one common spot on upper part of the elytra. M2 and M5 have irregular striped pattern that does not resemble any other type, and their cluster analysis showed that they differed more from the rest of the types phenotypically, as well as genotypically as they showed high genetic distance to the others types.

4. DISCUSSION

Since many factors can alter the reproducibility of RAPD PCR products and polymorphism analysis, it is

Table-4: Nei's Original Measures of Genetic Distance

Types of Species	1	2	3	4	5	6
M1	****					
M2	0.5108	****				
M3	0.393	0.393	****			
M4	0.5108	0.5978	0.393	****		
M5	0.5534	0.5534	0.5108	0.3216	****	
M6	0.5108	0.5978	0.2549	0.2231	0.6444	****

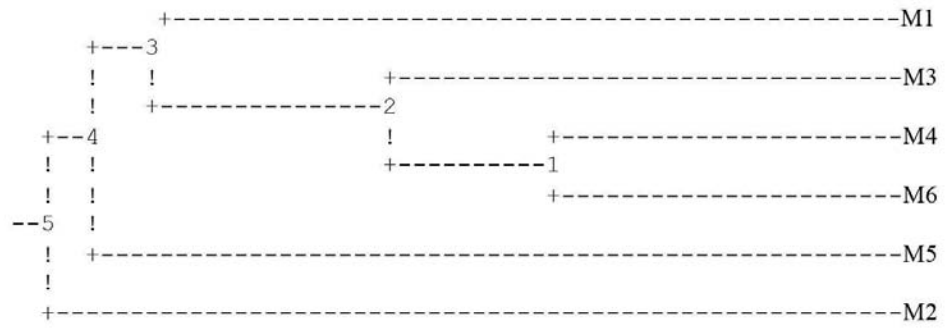

Figure-6: Dendogram Demonstrating the Relationship Among Six Types of *M. sexmaculatus* Based on RAPD PCR Data

necessary to standardize the amplification conditions prior to analysis of insect DNA, such as from *M. sexmaculatus*. It was found that each primer generated a variable number of amplified fragments, depending on the analyzed type. Generally, the proportion of polymorphic bands was higher than that of monomorphic bands. In this study, most of the tested primers yielded identical DNA bands among all types and were therefore not useful to differentiate these types into strains.

Bands of same size (360 bp, 380 bp, 500 bp, 650 bp, 680 bp) were observed among six types. It is generally recognized that bands of the same size may indicate homology among different types. However, it is well-documented that PCR products of the same size are not necessarily to be of the same DNA sequences. Therefore, those bands may have different DNA sequences and genetic makeup. Thus, DNA sequencing technique would be necessary to confirm the specificity of nucleotide sequences of similar bands. There were some differences in the DNA band profile within these types because of intraspecific polymorphism. Presence of strain specific fragments (880 bp in type M4 with OPA-04, 800 bp and 940 bp in type M5 with OPA-18, 380 bp in type M2 and 500 bp in type M5, 900 bp in type M5 and 1200 bp in type M3 with OPC-18) are indicative of heterogeneity among different types of *M. sexmaculatus*. In this study, we only considered the position of RAPD band chart but ignored the breadth and brightness of those bands, so this may lead to loss of some information. Besides, cluster analysis, some more accurate and reasonable methods, in which the position, breadth and brightness of bands should be considered, need to be developed in RAPD analysis.

A similar work was reported by Williams, et al. (1994) working with the *Weevil (L. bonariensis)* and Dowdy, et al. (1996) working with Indian meal moth in South America. But their data was not as clear because their working species strains exhibit a relatively high degree of genetic similarity with each other and the average banding difference between strains were not significant.

The analysis of geographical variation of polymorphism of the elytra in this species showed that although there were differences in the distribution of coloration pattern in different regions, these differences did not appear to be influenced by location variable factor analyzed here. This study demonstrated no association between location variability and the diversities. Almost all types or morphs were collected from one common location (AJK) and one other location (either Punjab or Sindh). Most closely related morphs were found to be located in different provinces and most distantly related

morphs were found to be located in the same area. The genetic diversity within sites might be influenced by population size in *M. sexmaculatus*; local population sizes are known to influence the degree of genetic variation within and between populations (Lande, 1995). Similar observations have been reported for *Plodia interpunctella* (Dowdy and Mc Gaughey, 1996).

Host variable factors were also analyzed to verify what influence they might be having on the diversity found in this species. The specimens of all types were collected from Maize, Rice and Cotton, and the result suggested that the polymorphism reported in this study is not related to the host variability as the most closely related types M 4 and M 6 were collected from different hosts. The reason for the polymorphism of elytral spot and color pattern still remains to be explained satisfactorily.

In this study, RAPD method revealed a large number of polymorphisms, which can be used as genetic markers in research involving species diagnosis, population differentiation and genetic fingerprinting. This study has successfully detected polymorphism in *M. sexmaculatus* and defined six different strains that are genotypically and phenotypically different. The results of this study have shown that the RAPD PCR analysis can be used in species and strain identification. This study proposed that RAPD should be integrated into classical identification methods since it is faster, more economical and reliable. This technique will likely offer more solid and certain results when identifying polymorphic species. However, this study encourages further research and analysis using large number of specimens, as well as markers to confirm these preliminary findings. Identifying the forces responsible for genetic differentiation within this species is important for practitioners of biological control. Further molecular studies of this six spotted ladybird can play a considerable role in studies of coccinellids ecology, evolutionary biology, cytology and biogeography.

Biopesticides offer a more sustainable solution to pest control than synthetic alternatives. These identified six strains of *M. sexmaculatus* could be of great economic importance in agro-ecosystem as they can be successfully employed in the biological control of plant harming aphids.

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